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Immunology

Blood group A cross-reacting epitope defined by monoclonal antibodies NCC-LU-35 and -81 expressed in cancer of blood group O or B individuals: Its identification as Tn antigen

(lung cancer/mucin/A-like antigen/α-*N*-acetylgalactosamine)

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ABSTRACT **Two monoclonal antibodies, NCC-LU-35 and NCC-LU-81, have been established after immunization of mice with membrane preparations of human lung cancer Lu65 tumor xenograft cells grown *in vivo* and intact cells cultured *in vitro*, respectively. These two antibodies react specifically with a majority of human adenocarcinomas, irrespective of the host's blood group ABO status, as well as with normal tissues and erythrocytes of blood group A individuals. The antigenicity is associated with a high molecular weight mucin-like glycoprotein separated by gel filtration of Lu65 tumor extracts. The epitope of the mucin-like glycoprotein has been identified as α-*N*-acetylgalactosaminyl residue directly linked *O*-glycosidically to serine or threonine residues of polypeptides. This epitope was serologically detected several years ago and given the name Tn. Our identification of the epitope is based on the following results: (*i*) The antigen is sensitive to α-*N*-acetylgalactosaminidase, but not to sialidase or α-fucosidase. (*ii*) Various mono- and difucosyl A determinants, either type 1 or type 2 chain, cross-react with both antibodies. (*iii*) The reactivity with both antibodies can be created by treatment of glycophorin A of normal erythrocytes with sialidase followed by β-galactosidase. (*iv*) *N*-[^{3}H]acetylgalactosamine can be released by galactose oxidase/NaB^{3}H$_4$ treatment from the Lu65 mucin-like glycoprotein but not from the mucin-like glycoprotein of normal colonic mucosa upon reductive β-elimination (alkaline borohydride treatment). The antigen may be one of the tumor-associated A cross-reacting antigens occurring in a wide variety of human adenocarcinomas of hosts belonging to all ABO blood groups.**

The recent development of antigen analysis with monoclonal antibodies has made possible the identification of a large variety of tumor-associated differentiation-dependent carbohydrate antigens bound to proteins (glycoproteins) as well as lipids (glycolipids) (1). The presence of A-like antigen in tumors of blood group O and B individuals has been a long-standing problem in classical tumor immunology (2, 3), although the chemical nature of A-like antigen has not been clearly identified. A-like antigen has been shown to be heterogeneous, because different types of antigens have been identified depending on the method and reagents applied in analysis (refs. 4–10; see *Discussion*).

Monoclonal antibodies NCC-LU-35 and NCC-LU-81 were established after immunization of mice with lung carcinoma Lu65 cells. The antigen defined by both antibodies is not expressed in various normal tissues of blood group B and O hosts, but it is strongly expressed in various adenocarcinomas derived from blood group B and O as well as A hosts. These antibodies react with both normal and tumor tissues of blood group A individuals, although the reactivity of NCC-LU-81 with normal A tissue is highly restricted, and only a very weak reactivity was observed. Since the incidence of expression of this antigen in O or B tumors is much higher than that of "incompatible A" defined by other A-specific antibodies (see *Discussion*), the chemical nature of the epitope defined by these antibodies is of great interest. This paper describes the general properties of these antibodies and the epitope they define.

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MATERIALS AND METHODS

Immunization and Establishment of Monoclonal Antibodies. NCC-LU-35 was established after immunization of BALB/c mice with cell membrane fractions prepared from human lung carcinoma Lu65 tumor xenograft grown in nude mice. Histologically, Lu65 tumor was a giant cell carcinoma of the lung. The membrane fraction was obtained by the method of Veltri *et al.* (11). NCC-LU-81 was established after immunization of BALB/c mice with cultured cells derived from Lu65 tumors. The fusion was performed with P3-X63-AG8-U1 cells (12) using polyethylene glycol 4000. The hybridomas were grown and selected as described (13) by immunohistological screening of small tissue sections of three types of lung cancers (adenocarcinoma, squamous cell carcinoma, and giant cell carcinoma) and normal lung tissues. Various other cancer tissues and normal tissues obtained from surgery and autopsy were also used for further immunohistological analysis. The autopsy tissues were obtained within 3 hr of death. Blood groups A, B, O and AB were examined by hemagglutination tests as well as histochemically. The procedures for immunohistological preparations were as described (13, 14).

Antigen Preparation. The Lu65 tumors grown in nude mice were extracted by homogenization with phosphate-buffered saline (P_i/NaCl; 10 mM sodium phosphate, pH 7.2/0.9% NaCl) containing 0.1% sodium azide, followed by centrifugation at 10,000 × *g* for 30 min. The supernatant was subjected to gel filtration on a Sepharose CL-6B column (2.5 × 90 cm) equilibrated with P_i/NaCl containing 0.1% sodium azide. Fractions (5 ml) were collected and examined by solid-phase radioimmunoassay as described (13). The mucin-like antigen reacting with both NCC-LU-35 and -81 was detected in the void volume fraction.

Reactivity of Lu65 Antigen with Monoclonal Antibodies After Treatment with Various Enzymes. The void volume fraction was diluted 1:20 with P_i/NaCl, and 100 μl per well was placed on microtiter plates (Dynatech, Alexandria, VA), left overnight at 4°C, and washed with P_i/NaCl. The protein concentration determined by fluorescausin (15) in the original void volume fraction was ≈20 μg/ml. The antigen adsorbed

on plastic surface was treated with 0.2 and 0.04 unit of sialidase per ml (Behringswerke, Marburg, West Germany), or 0.05 and 0.01 unit of α-fucosidase per ml, or α-*N*-acetylgalactosaminidase of *Charonia lampas* (Seikagaku Chemical, Tokyo). All enzymes contained 0.2 mM phenylmethylsulfonyl fluoride. One hundred microliters of each enzyme was added to each well and incubated at 37°C for 2 hr. Each well was washed with P_i/NaCl and antibody binding was determined as described (13).

Glycolipid Samples and Anti-Carbohydrate Antibodies. Monofucosyl type 1 chain A was obtained from gastric cancer MKN45 cell line (16). Glycolipids with monofucosyl type 2 chain A of various chain lengths (A^a, A^b, A^c) were obtained from human erythrocytes (17, 18). Difucosyl type 1 chain A was obtained from human intestine (19), and difucosyl type 2 chain A was obtained from dog intestine (20); both were donated by John McKibbin (Department of Biochemistry, University of Alabama, Birmingham, AL) and further purified as acetates as described (16). Globo-A (21) and type 3 chain A glycolipids (22) were obtained from human erythrocytes as described. The reactivity of glycolipids on solid-phase coated on plastic plates with various antibodies was determined as described (23). Antibodies HH1, which defines difucosyl A with both type 1 and type 2 chain; HH2, which defines difucosyl type 1 chain A (ALe^b); and HH3, which defines difucosyl type 2 chain A (ALe^y), were established in this laboratory (unpublished data). Antibodies HH4, which reacts preferentially with monofucosyl type 2 chain A as well as fucose-less A, and HH5, which reacts with type 3 chain A as well as type 4 chain A, were also established in this laboratory (unpublished data). AH16, which defines A determinant irrespective of its carrier, was established as described (15). Another nonspecific anti-A antibody was obtained from DAKO (Copenhagen). Anti-T antibody directed to Galβ1→3GalNAc was obtained from ChemBiomed (Edmonton, Alberta, Canada).

Reactivity of NCC-LU-35 or -81 Antibodies with Glycophorin A After Enzymatic Degradation. Glycophorin A (24) was purchased from Sigma (St. Louis, MO) and its solution in P_i/NaCl (10 μg/ml) was adsorbed onto Dynatech microtiter plates as described above. The plates were treated with 0.02 unit of sialidase per ml (*Clostridium perfringens* type X, Sigma) for 4 hr, followed by 1 unit of bovine liver β-galactosidase per ml (grade III, Sigma) overnight at 37°C in the presence or absence of 0.01–10 μg of D-galactonic acid γ-lactone per well (Pfanstiehl Chemical, Waukegan, IL), which is an inhibitor of β-galactosidase. These wells underwent reaction with antibody as described (23).

Demonstration of Base Unstable GalNAc Residue in Lu65 Glycoprotein Antigen and in Mucin Glycoproteins from Normal Mucosa. Mucin glycoproteins were prepared from surgically obtained normal colonic mucosa of a blood group B individual by the same method used for Lu65 glycoprotein antigen preparation. Labeling of Lu65 glycoprotein antigen and normal mucin glycoprotein with galactose oxidase and NaB^3H_4 was performed by incubation of 100 μg of glycoprotein dissolved in 200 μl of phosphate buffer (pH 7.0) containing 10 units of galactose oxidase (type V, 95 units/mg; Sigma) at room temperature for 4 hr, followed by addition of 10 μl of 1 M sodium borate ($Na_2B_4O_7$) and 0.5 mCi of NaB^3H_4 (1 Ci = 37 GBq) dissolved in 25 μl of 0.01 M NaOH. The mixture was allowed to stand at room temperature for 4 hr, then 25 μl of unlabeled 1% sodium borohydride ($NaBH_4$) was added, dialyzed overnight, and lyophilized. The residue was dissolved in 200 μl of water/100 μl of 1 M $Na_2B_4O_7$/80 μl of 1 M NaOH in a sealed tube and incubated at 37°C for 36 hr. This condition for reductive β-elimination is within the range suggested (25). The incubation mixture was treated with Dowex 50 (H^+ form) and filtered. The filtrates were lyophilized, and the residue was dissolved in methanol to form methylborate and evaporated to dryness under a nitrogen stream. The residue was dissolved in 100 μl of water, and 30-μl aliquots were run on paper chromatography on Whatman 3 MM paper in *n*-butanol/pyridine/water (6:4:3). *N*-acetylgalactosaminitol was run as a control and stained with silver nitrate/sodium hydroxide (25). The radioactivity released by alkaline borohydride degradation from Lu65 glycoprotein was ≈8.8×10^6 cpm per 100 μg of protein. The activity released from mucin glycoprotein from normal mucosa was 4.1×10^6 cpm per 100 μg of protein.

RESULTS

Immunohistological Reactivity of NCC-LU-35 and NCC-LU-81 Antibodies. Both antibodies strongly stained various lung cancers, particularly adenocarcinomas, and other cancers from stomach, pancreas, and breast, irrespective of the host's blood group status. The NCC-LU-35 antibody reacted with normal lung tissue of blood group A and AB hosts, whereas the NCC-LU-81 antibody reacted only weakly. Neither of the antibodies reacted with normal lung tissue of O and B hosts. Details of immunohistological staining of various normal and tumor tissues will be described elsewhere. A few typical staining patterns of normal lung and lung cancer tissues are shown in Fig. 1, and the incidence of positive staining with NCC-LU-35 and -81 antibodies of various cancer tissues from blood group A and non-A

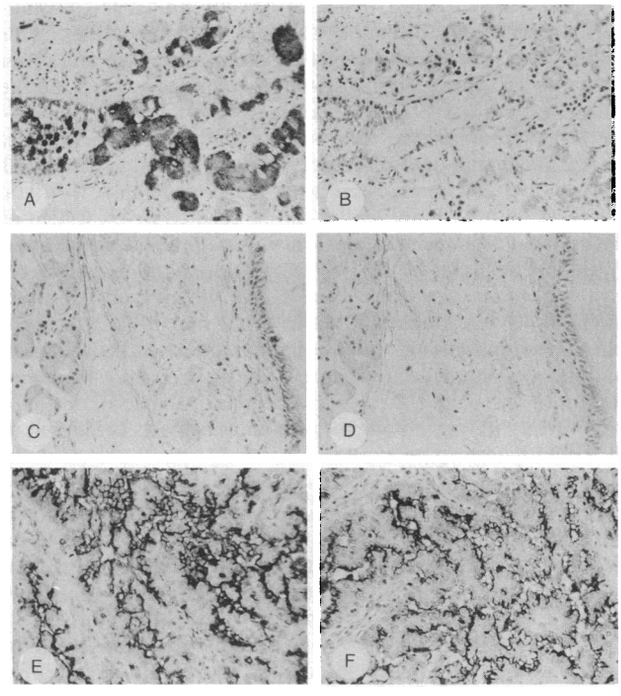

FIG. 1. Immunoperoxidase staining pattern of normal lung and lung cancer tissue. (*A*) Normal lung tissue from blood group A individual stained by NCC-LU-35 (mucinous material present in bronchial glands is stained). (*B*) The same as in *A*, stained by NCC-LU-81 (no staining). (*C*) Normal lung tissue from blood group O individual stained by NCC-LU-35. (*D*) The same as in *C*, stained by NCC-LU-81. (Both not stained). (*E*) Adenocarcinoma of lung from blood group O individual stained by NCC-LU-35. (*F*) The same as in *E*, stained by NCC-LU-81. (Both stained strongly at cell surface.) Sections were prepared from formalin-fixed paraffin-embedded blocks. The staining was performed with culture supernatant of hybridomas, biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA), and avidin–biotin periodate complex (Vector Laboratories). Finally, the sections were developed with diaminobenzidine and were counter-stained with Mayer's hematoxylin (14).

Table 1. Immunohistochemical reactivity of monoclonal antibodies NCC-LU-35 and NCC-LU-81 with tissue sections from various human cancers with different host blood types

Cancer type	NCC-LU-35		NCC-LU-81		Anti-A (DAKO)	
	A and AB	B and O	A and AB	B and O	A and AB	B and O
Lung adenocarcinoma	16/18	11/17	10/18	9/17	15/18	1/17
Squamous	5/6	5/8	1/6	4/8	3/6	0/8
Large	8/16	11/18	2/16	8/18	5/16	0/18
Small	0/8	0/8	0/8	0/8	0/8	0/8
Pancreas	11/11	11/13	7/11	8/13	9/11	2/13
Breast	4/4	5/6	4/4	5/6	1/4	1/6
Colon	4/4	6/7	3/4	5/7	3/4	1/7
Stomach	41/41	42/44	34/41	41/44	40/41	4/44

individuals is shown in Table 1. As clearly indicated, blood group B and O tumors showed a large amount of positive staining by both antibodies as compared with the lesser amount of positive staining by DAKO's anti-blood group A antibody, which is specific to trisaccharide A structure GalNAcα1→3(Fucα1→2)Gal. The two antibodies showed similar specificities, although the reactivity of NCC-LU-81 was more restricted than that of NCC-LU-35, particularly with normal A tissue.

The Reactivity of the Mucin-Like Glycoprotein Antigen Isolated from Lu65 Tumors with NCC-LU-35 and -81, Anti-T, and Various Anti-A Antibodies. The mucin-like antigen isolated from Lu65 tumors was found to react strongly with both NCC-LU-35 and -81 antibodies on solid-phase radioimmunoassay (Figs. 2 and 3*A*). The reactivity on solid phase was greatly decreased or nearly abolished by treatment with α-*N*-acetylgalactosaminidase but not sialidase or α-L-fucosidase (Fig. 2). The solid-phase antigen from Lu65 tumors was also highly reactive with anti-T antibody and weakly reactive with HH4 antibody (Fig. 3*B*). Four other anti-A monoclonal antibodies were barely reactive (Fig. 3*B*).

The Reactivity of Blood Group A Glycolipids with NCC-LU-35 and -81 Antibodies. The reactivities of NCC-LU-35 and -81 on solid-phase radioimmunoassay with various blood group A glycolipids, including mono- and difucosyl type 1 and type 2 chain A, type 3 chain A, and globo-A, are shown in Fig. 3*C* and *D*, respectively. As indicated, both antibodies cross-reacted strongly with various A determinants, except type 3 chain A and globo-A. The reactivity was less restricted with NCC-LU-35 antibody than with NCC-LU-81.

The Reactivity of Glycophorin A with NCC-LU-35 and -81 Antibodies Before and After Enzymatic Treatment. Treatment of solid-phase glycophorin A adsorbed on plastic surface with sialidase followed by bovine liver β-galactosidase greatly increased the reactivity of glycophorin with both antibodies (Fig. 4 *A–C*). The appearance of reactivity with both antibodies upon treatment of solid-phase glycophorin with β-galactosidase was inhibited by D-galactonic acid γ-lactone. With increasing concentration of D-galactonic acid γ-lactone, the inhibition of reactivity was increasingly apparent (Fig. 4*D*).

Release of GalNAc Residue from Mucin Glycoprotein of Lu65 Tumors But Not from Normal Mucin Glycoprotein by Reductive β-Elimination. A strong peak corresponding to *N*-[^{3}H]acetylgalactosaminitol was released from Lu65 glycoprotein when the glycoprotein was labeled with galactose oxidase and NaB^3H_4 and treated with alkaline $NaBH_4$. Two other smaller peaks with slower chromatographic mobilities than *N*-acetylgalactosaminitol were also released from Lu65 glycoprotein. No label corresponding to *N*-acetylgalactosaminitol or other oligosaccharides was detected by the same treatment of labeled glycoprotein from normal tissues. Only a high molecular weight substance with a very slow mobility was labeled in normal tissues (Fig. 5).

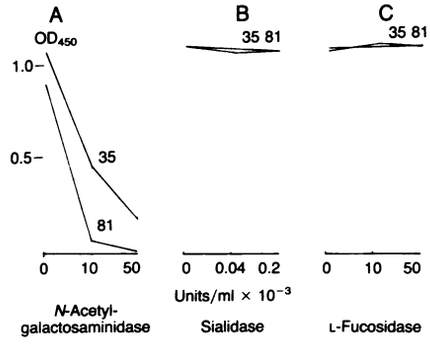

FIG. 2. Reactivity of monoclonal antibodies NCC-LU-35 and NCC-LU-81 to solid-phase mucin glycoprotein extracted from Lu65 tumor. Glycoprotein fraction (100 μl per well) purified on gel filtration and diluted 1:20 was placed in Dynatech microtiter plates and incubated overnight to effect adsorption of the antigen. The plates were washed and incubated with various concentrations of enzymes as indicated. The antibody binding was determined by ELISA as described.

DISCUSSION

The monoclonal antibodies NCC-LU-35 and -81 were selected on the basis of specific immunohistologic reactivity with human cancer tissues, irrespective of host's blood group ABO status, and negative reactivity with normal tissues. Antibody NCC-LU-35 showed preferential reactivity with tissues and erythrocytes from blood group A individuals, but NCC-LU-81 did not. However, both antibodies showed a cross-reactivity with isolated blood group A glycolipid antigens over other types of antigens, indicating that the epitope defined by these antibodies partially overlaps with A antigen.

Both NCC-LU-35 and -81 antibodies showed a strong reactivity with a mucin-like glycoprotein isolated from human lung cancer Lu65 tumor xenograft grown in nude mice. The epitope recognized by these two antibodies has been identified as α-*N*-acetylgalactosaminyl residue *O*-glycosidically linked to serine or threonine residue of polypeptides, based on the following results: (*i*) The activity of the cellular extract of Lu65 was associated exclusively with mucin-type glycoproteins, and the activity was abolished by α-*N*-acetylgalactosaminidase treatment. No reactivity was observed with glycolipid preparations extracted from 10 cases of human cancers. (*ii*) Only the highly active mucin glycoprotein of Lu65 tumors, but not the inactive normal glycoprotein, released *N*-[^{3}H]acetylgalactosaminitol after being labeled with galactose oxidase/NaB^3H_4 and reductive β-elimination. (*iii*) Inactive glycophorin A became highly active after sialidase treatment followed by treatment with bovine liver β-galactosidase. Other β-galactosidases from various sources were incapable of cleaving the β-galactose residue of Galβ1→GalNAcα→Cer. (*iv*) Both antibodies cross-reacted with type 1 or type 2 chain A glycolipids because of the presence of αGalNAc residue, but not with type 3 chain A nor

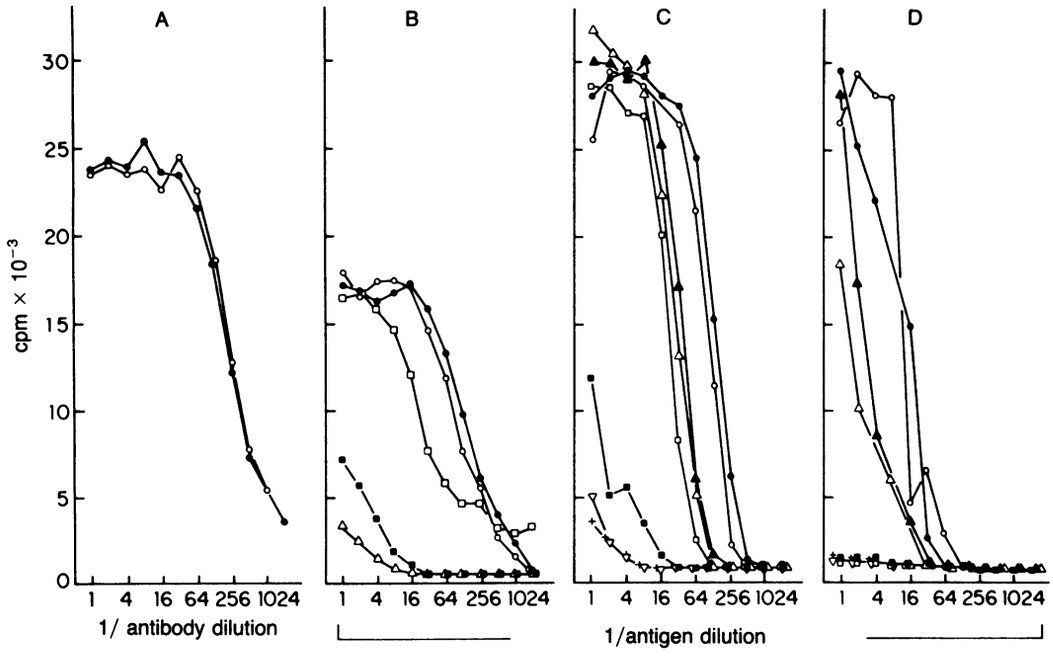

FIG. 3. Reactivity of glycoprotein extracted from Lu65 tumor (Lu65 antigen) and purified blood group A glycolipids having different carrier structures with various antibodies. (*A*) Binding of antibody LU-35 (●) and LU-81 (○) to solid-phase Lu65 antigen. The binding was determined with different concentrations of antibody to constant concentration of antigen (antigen solution diluted 1:25 and incubated as described in Fig. 2 legend). (*B*) Reactivity of various antibodies to various concentrations of Lu65 antigen adsorbed on solid phase. Antibodies applied were LU-35 (●), LU-81 (○), anti-T (□), HH4 (■), and four other anti-A monoclonal antibodies (HH1, HH2, HH3, and HH5) that showed insignificant reactivity (△). (*C*) Reactivity of LU-35 with various glycolipids on solid phase at different concentrations. △, A^b; ▲, type 1 chain A^a; □, ALe^b; ○, A^a; ●, ALe^y; ■, type 3 chain A; ▽, globo-A; +, Forsmann. Undiluted concentration of glycolipids applied was 200 ng per well. (*D*) Reactivity of LU-81 with various glycolipids. Symbols for each glycolipid are the same as in *C*. Antibodies in *C* and *D* were culture supernatants diluted 1:1. Anti-T antibody in *B* was diluted 1:1000.

globo-A. ALe^b did not react with NCC-LU-81. On the other hand, various anti-A antibodies that define trisaccharide structure GalNAcα1→3(Fucα1→2)Gal did not react with the Lu65 antigen.

Incompatible A antigen has been found in 10–20% of blood group O tumors, and some of the incompatible A antigen has now been chemically identified as mono- or difucosyl type 1 chain A, as clearly defined by specific monoclonal antibodies directed to these structures (9). Nonetheless, Forssman antigen (10) and fucose-less A antigen (7) may also be detected if the proper methodology and reagents are applied. The epitope to which antibodies NCC-LU-35 and -81 are directed is clearly different from those of "incompatible A antigen," which is expressed in O tumors with a much lower incidence than the antigen defined by NCC-LU-35 and -81 antibodies. An A-like antigen highly expressed in O or B tumors described by Häkkinen (5) may well be the same type of antigen defined by NCC-LU-35 and -81.

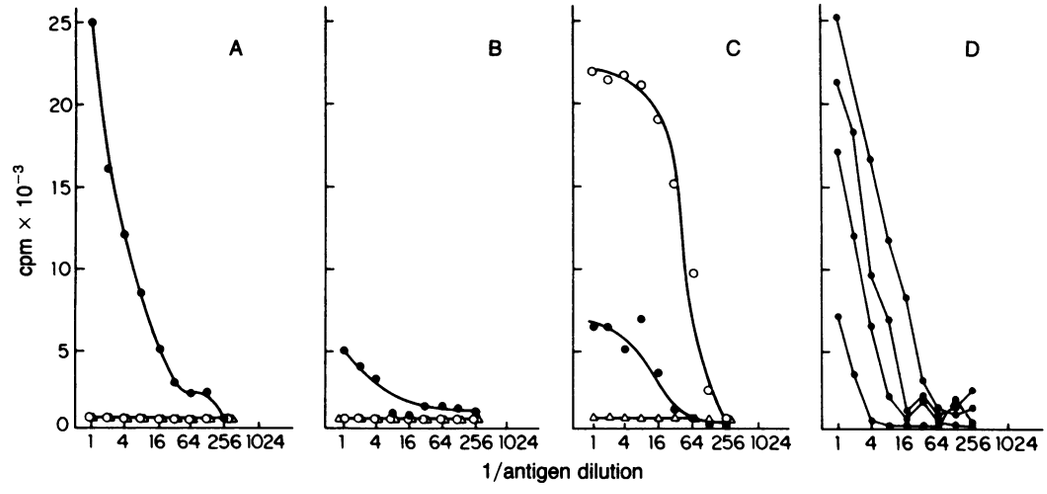

FIG. 4. Reactivity of glycophorin A of human erythrocytes with antibodies LU-35, LU-81, and anti-T before and after enzymatic degradation. (*A* and *D*) LU-35; (*B*) LU-81; (*C*) anti-T. △, untreated glycophorin A; ○, sialidase-treated glycophorin A; ●, treated with sialidase and subsequently with β-galactosidase. (*D*) Inhibition of LU-35 binding to solid-phase sialidase- and β-galactosidase-treated glycophorin observed by coincubation of D-galactonic acid γ-lactone and β-galactosidase (Sigma). Four curves indicate the inhibition by 10 μg, 1 μg, 0.1 μg, and 0.01 μg of D-galactonic acid γ-lactone per well.

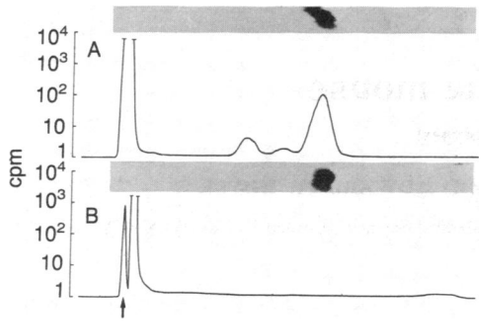

FIG. 5. Radiochromatogram of *N*-acetylgalactosaminitol and other oligosaccharides released from mucin-like glycoprotein of Lu-65 tumor but not from normal colonic mucosa. Each 100 μg of mucin-like glycoprotein isolated from Lu65 tumor extracts and from normal colonic mucosae was labeled with galactose oxidase/NaB^3H_4 followed by alkaline borohydride degradation, and radioactive mono- and oligosaccharides, separated on paper chromatography, were scanned by Packard radiochromatography. Ordinate indicates the radioactivity, and abscissa indicates the time (1 cm/min). Guide strips containing *N*-acetylgalactosaminitol were stained with silver nitrate/sodium hydroxide.

The A-like antigenicity of αGalNAc residue linked to serine or threonine has been described by Uhlenbruck and associates as the Tn antigen, detectable by various GalNAc lectins (*Helix pomatia, Soja hispida,* and *Sarothamnus scoparius*) that preferentially agglutinate blood group A erythrocytes (26). The antigen is cryptic in normal glycoproteins, but becomes exposed after desialylation followed by Smith degradation. Direct Smith degradation of glycoproteins does not expose cryptic Tn (26). The Tn antigen has also been described by Springer and associates as the precursor of the Thomsen–Friedenreich antigen (T antigen), and both Tn and T antigens are expressed in breast cancer tissue (27). More recently, Springer and associates observed a weak anti-Tn antibody in normal human antisera and applied it in the detection of the cancer-associated Tn antigen by immunohistology and absorption assay (28). They claimed that Tn antigen expression (in terms of absorption titer) in highly metastatic tumors was greater than in less metastatic differentiated tumors. The results of our present study, using well-defined highly specific high-titer monoclonal antibodies, support those observations. The exposure of Tn antigen in various blood cell components has also been observed under a certain acquired human nonmalignant condition resulting from a somatic mutation occurring at the level of bone marrow stem cells and termed "Tn syndrome" (26, 29). Tn-positive cells are deficient in β1→3 galactosyltransferase to αGalNAc linked to protein (30).

The exposure of Tn antigen in tumor-associated glycoprotein may indicate the importance of the incomplete synthesis of carbohydrate chains associated with oncogenesis. The concept was originally based on glycolipid changes in oncogenically transformed cultured cells (31) as well as *in vivo* tumors (32). Although "neosynthesis" rather than incomplete synthesis predominates in various lacto-series carbohydrate chains in both glycolipids and glycoproteins (32), some peripheral side chains in *N*-linked oligosaccharides show incomplete synthesis as well (33). The appearance of Tn antigen, as shown in this study, could be the result of incomplete synthesis of a major *O*-linked sugar chain in mucin glycoprotein, which is caused by a block in sialyltransferase or β-galactosyltransferase associated with human oncogenesis.

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